

APPLICATION FOR UNITED STATES LETTERS PATENT

for

FULLERENES IN TARGETED THERAPIES

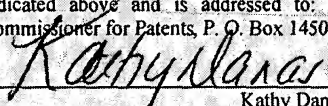
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BACKGROUND OF THE INVENTION

The present application claims priority from Serial No. 60/398,325, filed July 24, 2002.

5 **1. Field of the Invention**

The present invention relates generally to the field of targeted therapies. More particularly, it concerns compositions comprising fullerenes or nanotubes associated with antibodies or targeting peptides, and optionally further associated with therapeutic molecules.

10

2. Description of Related Art

In general, “targeted therapies” are therapeutic compositions and methods that involve the delivery of a drug to a particular diseased cell, tissue, organ, or organ system in a mammal, such as a human or a mammal providing esthetic, economic, or research
15 benefits to humans. The benefits of a targeted therapy include the potential to use less of the drug and the minimizing of systemic side effects the drug may have on the mammal.

Antibodies, including monoclonal antibodies, and targeting peptides are known which engage in highly specific physical interactions with particular antigens.

Linking a therapeutic molecule directly to an antibody or targeting peptide has
20 been attempted, but has encountered a number of difficulties. Frequently, the antibody or targeting peptide has multiple sites at which reaction with the therapeutic molecule can occur, and vice versa, and reaction at some of those sites can lead to changes in the drug’s or the antibody or targeting peptide’s structure and potentially impair its function. Also, if the therapeutic molecule’s and the antibody or targeting peptide’s active sites are
25 free to move relative to each other (while remaining linked), the therapeutic molecule and the antibody or targeting peptide may interact physically or chemically (through favorable van der Waals interactions, hydrophobic interactions, hydrogen bonding, or ionic association, among others) and adopt conformations that change the structure and potentially impair the functions of the drug, the antibody or targeting peptide, or both.

Therefore, a need exists for improved compositions for use in targeted therapies.

Fullerenes, of which the best known example is C₆₀, were first reported by Kroto et al., *Nature* (1985) 318:162. Since then, the ready derivatization of fullerenes has allowed a wide variety of derivatized fullerenes to be prepared and their properties explored.

Amphiphilic derivatized fullerenes have been reported by Hirsch et al., *Angew. Chem. Int. Ed.* (2000) 39(10):1845-1848. The derivatized fullerenes of Hirsch comprised one dendrimeric group comprising 18 carboxylic acid moieties and five hydrophobic moieties each comprising a pair of lipophilic C₁₂ hydrocarbon chains. Freeze-fracture electron micrography of aqueous solutions of the amphiphilic derivatized fullerenes revealed that the amphiphilic derivatized fullerenes formed bilayer vesicles (by which is meant, a vesicle defined by a membrane comprising an external layer of amphiphilic derivatized fullerene molecules substantially all oriented with their hydrophilic groups to the exterior of the vesicle, and an internal layer of amphiphilic derivatized fullerene molecules substantially all oriented with their hydrophilic groups to the interior of the vesicle, wherein the hydrophobic groups of the molecules of the external layer are in close proximity to the hydrophobic groups of the molecules of the internal layer) with diameters from about 100 nm to about 400 nm.

Carbon nanotubes and methods for their derivatization are known. Holzinger et al., *Angew. Chem. Int. Ed.* (2001) 40(21):4002-4005 report the cycloaddition of nitrenes, the addition of nucleophilic carbenes, and the addition of radicals, to the sidewalls of carbon nanotubes.

SUMMARY OF THE INVENTION

In one embodiment, the present invention relates to a composition comprising a C_n-Ab, wherein C_n is a fullerene or nanotube comprising n carbon atoms, Ab is a moiety comprising an antigen-binding site and is linked to the C_n, a therapeutic molecule is associated with the C_n, and the therapeutic molecule comprises a radioisotope.

In another embodiment, the present invention relates to a method of treating a disease in a mammal, comprising administering to the mammal an effective amount of a composition comprising (i) a C_n -Ab, wherein C_n is a fullerene or nanotube comprising n carbon atoms, Ab is a moiety comprising an antigen-binding site and is linked to the C_n , a therapeutic molecule is associated with the C_n , and the therapeutic molecule comprises a radioisotope, and (ii) a pharmaceutically-acceptable carrier.

In certain embodiments, the composition provides a fullerene or nanotube core which allows precise localization of linking reactions and provides a fixed structure which minimizes the freedom of movement of the Ab and a therapeutic molecule relative to each other, thus minimizing the drawbacks discussed above.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

Figure 1 shows a schematic representation of a C_n -Ab conjugate associated by a noncovalent antibody-mediated interaction.

Figure 2 shows a schematic representation of a C_n -Ab conjugate associated by a covalent linker.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

In one embodiment, the present invention relates to a composition, comprising: a C_n -Ab, wherein C_n is a fullerene or nanotube comprising n carbon atoms, and Ab is a moiety comprising an antigen-binding site and is linked to the C_n .

C_n refers to a fullerene moiety comprising n carbon atoms or a nanotube moiety comprising at least n carbon atoms. Buckminsterfullerenes, also known as fullerenes or, more colloquially, buckyballs, are closed-cage molecules consisting essentially of

sp^2 -hybridized carbons. Fullerenes are the third form of pure carbon, in addition to diamond and graphite. Typically, fullerenes are arranged in hexagons, pentagons, or both. Most known fullerenes have 12 pentagons and varying numbers of hexagons depending on the size of the molecule. Common fullerenes include C_{60} and C_{70} (*i.e.*, $n =$
5 60 or $n = 70$), although fullerenes comprising up to about 400 carbon atoms are also known.

Single-wall carbon nanotubes, also known as single wall tubular fullerenes, are cylindrical molecules consisting essentially of sp^2 -hybridized carbons. In defining the size and conformation of single-wall carbon nanotubes, the system of nomenclature
10 described by Dresselhaus et al., *Science of Fullerenes and Carbon Nanotubes*, Ch. 19, *ibid.* will be used. Single wall tubular fullerenes are distinguished from each other by a double index (x, y) , where x and y are integers that describe how to cut a single strip of hexagonal graphite such that its edges join seamlessly when the strip is wrapped onto the surface of a cylinder. When $x = y$, the resultant tube is said to be of the "arm-chair" or
15 (x, x) type, since when the tube is cut perpendicularly to the tube axis, only the sides of the hexagons are exposed and their pattern around the periphery of the tube edge resembles the arm and seat of an arm chair repeated n times. When $y = 0$, the resultant tube is said to be of the "zig zag" or $(x, 0)$ type, since when the tube is cut perpendicular to the tube axis, the edge is a zig zag pattern. Where $x \neq y$ and $y \neq 0$, the resulting tube
20 has chirality. The electronic properties of the nanotube are dependent on the conformation, for example, arm-chair tubes are metallic and have extremely high electrical conductivity. Other tube types are metallic, semi-metals or semi-conductors, depending on their conformation. Regardless of tube type, all single-wall nanotubes have extremely high thermal conductivity and tensile strength.

25 The single wall carbon nanotube can be a cylinder with two open ends, a cylinder with one closed end, or a cylinder with two closed ends. Generally, an end of a single wall carbon nanotube cylinder can be closed by a hemifullerene, *e.g.*, a $(10, 10)$ carbon nanotube can be closed by a 30-carbon hemifullerene. If the single wall carbon nanotube has one or two open ends, the open ends can have any valences unfilled by carbon-carbon
30 bonds within the single wall carbon nanotube filled by bonds with hydrogen, hydroxyl

groups, carboxy groups, or other groups. In one embodiment, the unfilled valences are filled by bonds with -COOH, or a salt or ester thereof.

5 In one embodiment, the C_n can be a fragment of a fullerene or a fragment of a single wall carbon nanotube. A fragment of a single wall carbon nanotube can be generated by fluorination and subsequent pyrolysis, and a fragment of a desired size can be isolated by size separation by any appropriate technique, such as size-exclusion chromatography, nanopore filtration, capillary electrophoresis, or centrifugation, among others. In one embodiment, the fragment of a single wall carbon nanotube is about 20 nm in length.

10 In another embodiment, the C_n is not a fragment of a fullerene or a fragment of a single wall carbon nanotube.

The value of n can be other than a value explicitly stated herein and remain within the scope of the present invention.

15 The C_n can be substituted or unsubstituted. By “substituted” is meant that a group of one or more atoms is covalently linked to one or more atoms of the C_n . Exemplary groups include, but are not limited to, malonate groups and groups derived from malonate, among others. In one embodiment, the C_n is substituted with one or more water-solubilizing groups. Water-solubilizing groups are polar groups (that is, groups having a net dipole moment) that render the generally hydrophobic fullerene core soluble in water.

20 A powerful observation regarding fullerenes and nanotubes is that they generally allow for precise localization of substituent molecules during the addition of substituents thereto.

25 Ab refers to a moiety comprising an antigen-binding site. An “antigen,” as used herein, is a chemical compound or a portion of a chemical compound which can be recognized by a specific chemical reaction, a specific physical reaction, or both with another molecule. The antigen-recognition site of an antibody is an exemplary, but non-limiting, antigen-binding site. Examples of moieties comprising antigen-binding sites include, but are not limited to, monoclonal antibodies, polyclonal antibodies, Fab fragments of monoclonal antibodies, Fab fragments of polyclonal antibodies, Fab₂

fragments of monoclonal antibodies, and Fab₂ fragments of polyclonal antibodies, among others. Single chain or multiple chain antigen-recognition sites can be used. Multiple chain antigen-recognition sites can be fused, joined by a linker, or unfused and unlinked.

5 The Ab can be selected from any known class of antibodies. Known classes of antibodies include, but are not necessarily limited to, IgG, IgM, IgA, IgD, and IgE. The various classes also can have subclasses. For example, known subclasses of the IgG class include, but are not necessarily limited to, IgG1, IgG2, IgG3, and IgG4. Other classes have subclasses that are routinely known by one of ordinary skill in the art.

10 The Ab can be derived from any species. "Derived from," in this context, can mean either prepared and extracted *in vivo* from an individual member of a species, or prepared by known biotechnological techniques from a nucleic acid molecule encoding, in whole or part, an antibody peptide comprising invariant regions which are substantially identical to antibodies prepared *in vivo* from an individual member of the species or an antibody peptide recognized by antisera specifically raised against antibodies from the
15 species. Exemplary species include, but are not limited to, human, chimpanzee, baboon, other primate, mouse, rat, goat, sheep, and rabbit, among others known in the art. In one embodiment, the Ab is chimeric, *i.e.*, comprises a plurality of portions, wherein each portion is derived from a different species. A chimeric antibody, wherein one of the portions is derived from human, can be considered a humanized antibody.

20 Abs are available that recognize antigens associated with a wide variety of cell types, tissues, and organs, and a wide variety of medical conditions, in a wide variety of mammalian species. Exemplary medical conditions include, but are not limited to, cancers, such as lung cancer, oral cancer, skin cancer, stomach cancer, colon cancer, nervous system cancer, leukemia, breast cancer, cervical cancer, prostate cancer, and
25 testicular cancer; arthritis; infections, such as bacterial, viral, fungal, or other microbial infections; and disorders of the skin, the eye, the vascular system, or other cell types, tissues, or organs; among others.

Exemplary Abs include, but are not limited to, those derived from antibodies against vascular endothelial growth factor receptor (VEGF-r) (available from Imclone,
30 New York, NY), antibodies against epidermal growth factor receptor (EGF-r) (available

from Abgenix, Fremont, CA), antibodies against polypeptides associated with lung cancers (available from Corixa Corporation, Seattle, WA), and antibodies against human tumor necrosis factor alpha (hTNF- α) (available from BASF A.G., Ludwigshafen, Germany), among others known in the art.

5 Abs can be prepared by various techniques known in the art. These techniques include, but are not limited to, the immunological technique described by Kohler and Milstein in Nature 256, 495-497 (1975) and Campbell in "Monoclonal Antibody Technology, The Production and Characterization of Rodent and Human Hybridomas" in Burdon et al., Eds., Laboratory Techniques in Biochemistry and Molecular Biology,
10 Volume 13, Elsevier Science Publishers, Amsterdam (1985); as well as by the recombinant DNA techniques described by Huse et al in Science 246, 1275-1281 (1989); among other techniques known to one of ordinary skill in the art.

 In one embodiment, the Ab is derived from murine ZME-018, which recognizes the gp240 antigen present on more than 80% of melanoma biopsies and cell lines. The
15 gp240 antigen can also be recognized by Abs derived from SCFVMEL, an SCFV antibody; dSCFVMEL, a diabody antibody; and GD2, a chimeric antibody. In another embodiment, the Ab is derived from HuM195, a humanized antibody which recognizes CD-33, an antigen present on AML and CML cells in the hematopoic system. In a different embodiment, the Ab is derived from herceptin, a chimeric antibody which
20 recognizes the HER2 antigen associated with certain breast, colon, and lung tumors. The HER2 antigen can also be recognized by Abs derived from the BACH 250 chimeric antibody, the ML 3-9 SCFV antibody, or the C 6.5 diabody antibody. In another embodiment, the Ab is derived from α MMP9, a chimeric antibody which recognizes the MMP9 antigen associated with certain lung tumors. In a different embodiment, the Ab is
25 derived from Campath 1H, an antibody which recognizes the CD-52 antigen associated with leukemias. In a further embodiment, the Ab is derived from anti-TNF-R1, an antibody which recognizes the TNF-R1 antigen associated with leukemias. In yet a different embodiment, the Ab is derived from anti-CD-38, an antibody which recognizes the CD-38 antigen associated with leukemias. In still a further embodiment, the Ab is
30 derived from Bexxar, an antibody which recognizes the CD-20 antigen associated with

leukemias. In still an additional embodiment, the Ab is derived from VEGF121 or SuperGen, antibodies which recognize the VEGF Receptor 2 antigen associated with solid tumors.

5 In addition to the listed antibodies, the Ab can be constructed to recognize a target antigen associated with a solid tumor. For example, the Ab can be constructed to recognize HER2/neu, MUC-1, HMFG1, or EGFr, associated with breast tumors; MMP-9, HER2/neu, or NCAM, associated with lung tumors; HER2 or 171A, associated with colon tumors; gp240, gangliosides, or integrins, associated with melanomas; HER2 or CA-125, associated with ovarian tumors; or EGFr or tenascin, associated with brain
10 tumors. This list of target antigens and tumor types is exemplary and not limiting.

In addition the antigen-binding site, the Ab can comprise a linker. The linker can be any moiety covalently bound to the portion of the Ab containing the antigen-binding site and capable of associating with the C_n . In one embodiment, the association involves a specific physical interaction between the linker and the C_n . For example, the linker can
15 be an antibody raised against the C_n to be used in the composition. An example of this embodiment is schematically represented in Figure 1, wherein one or more C_{60} fullerenes **10** are linked to an antitumor antibody **20** by an anti- C_{60} antibody **30** associated with the antitumor antibody **20** by covalent or noncovalent interactions. In another embodiment, the association can be covalent. The linker can be formed by, for example, (i)
20 substituting the C_n with a sulfhydryl-containing (-SH) substituent; (ii) preparing an Ab with a sulfhydryl-containing linker; and (iii) reacting the Ab and the C_n to form a -S-S-bond between the Ab and the C_n . An example of this embodiment is schematically represented in Figure 2, wherein a C_{60} fullerene **40** is linked to an antitumor antibody **50** via a disulfide bond **60**.

25 In addition to the antibodies described above, any other compound that can recognize a specific antigen can be used as the Ab described herein. Such other compounds include antibody fragments and certain synthetic peptides that are known or are discovered to recognize specific antigens, as well as other targeting bioagents. Such other compounds can further comprise linkers, as described above.

In addition to the C_n-Ab, the composition can further comprise one or more other compounds. In one embodiment, the composition can further comprise a pharmaceutically-acceptable carrier. A “pharmaceutically-acceptable carrier” is a compound in which the C_n-Ab can be dissolved, suspended, emulsified, mixed, or otherwise combined with. Further, the carrier is generally safe when administered to a mammal. Exemplary pharmaceutically-acceptable carriers include, but are not limited to, water, buffered aqueous solutions, sucrose, and gelatin, among others.

As stated above, the composition can further comprise a therapeutic molecule associated with the C_n-Ab. As used herein, a “therapeutic molecule” comprises a radioisotope, by which is meant an atom capable of α- or β-emission. Such atoms are capable of emitting radiation that is capable of killing diseased cells, such as tumor cells. In one embodiment, the radioisotope can be ¹²⁵I, ¹³¹I, ⁹⁰Y, ²²¹At, ²²⁵Ac, ²¹²Bi, ²¹³Bi, ⁹⁹Re, ¹⁶⁶Ho, ¹⁷⁷Lu, or ¹⁵³Sm, among others. The therapeutic molecule can comprise a plurality of radioisotopes. In one embodiment, the therapeutic molecule is an atom of the radioisotope.

One or more therapeutic molecules can be used in any composition or method of the present invention. If a therapeutic molecule comprises a radioisotope and two or more therapeutic molecules are used, other therapeutic molecules can comprise the same radioisotope or different radioisotopes, or can be non-radioisotopic therapeutic molecules, such as those described above.

The therapeutic molecule can be associated with the C_n-Ab by one or more routes of association. The association can be a covalent link between the fullerene or nanotube core and the therapeutic molecule; a covalent link between a substituent of the fullerene or nanotube, if any, and the therapeutic molecule; a non-covalent favorable van der Waals interaction; an ionic association between a positively- or negatively-charged group on a substituent of the fullerene or nanotube and an oppositely-charged group on the therapeutic molecule; or the encapsulation of the therapeutic molecule in the fullerene or nanotube core, among others. Encapsulation of the therapeutic molecule (M) in the fullerene or nanotube core (C_n) can be referred to as “M@C_n,” and when the C_n is linked with an Ab, the overall structure can be referred to as “M@C_n-Ab.”

A covalent link can be direct or it can make use of a covalent linker linking the therapeutic molecule and the fullerene or nanotube core or substituent, if any, of the fullerene or nanotube. In one embodiment, the covalent link can be cleavable by an appropriate cleaving technique, such as photolysis, enzymatic cleavage, or chemical cleavage, among others. In another embodiment, a non-covalent association between the therapeutic molecule and the fullerene or nanotube can be dissociated by application of an appropriate chemical, *e.g.*, when the association is an ionic association, the association can be dissociated by application of a charged compound of the same charge as the charged group on the therapeutic molecule. Other techniques for dissociating a non-covalent association between the substituted fullerene or nanotube and the therapeutic molecule will be apparent to one of ordinary skill in the art in light of the present specification. A chemical or enzyme used to promote dissociation can be referred to as an "adjuvant." In another embodiment, the therapeutic molecule is covalently linked to the fullerene or nanotube and is not dissociated therefrom.

In one embodiment, wherein the therapeutic molecule comprises a radioisotope, the therapeutic molecule can be encapsulated in the fullerene or nanotube core. "Encapsulated," as used herein, refers to either complete encagement of the therapeutic molecule by an intact fullerene or a nanotube with both ends closed, or partial encagement mediated by van der Waals or other favorable covalent or non-covalent interaction. In one embodiment, the nanotube core is an open-ended nanotube fragment and the therapeutic molecule is encaged within the nanotube fragment.

Any compound that can be associated with the C_n and that would be expected to have a therapeutic benefit when delivered to a specific tissue or cell type is a therapeutic molecule within the scope of the present claims. Whether a compound is expected to have a therapeutic benefit will depend on the specific tissue or cell type; the Ab attached to the C_n ; and other factors apparent to one of ordinary skill in the art.

If a therapeutic molecule is present, the order in which the therapeutic molecule and the Ab are associated with the C_n is not crucial.

The composition can further comprise one or more additional compounds not enumerated above, whose inclusion in compositions comprising C_n -Ab or useful in the

methods described below is a matter of routine experimentation for an ordinary skilled artisan having the benefit of the present disclosure.

5 In another embodiment, the present invention relates to a method of treating a disease in a mammal, comprising:
administering to the mammal an effective amount of a composition comprising (i) a C_n -Ab, wherein C_n is a fullerene or nanotube comprising n carbon atoms, and Ab is a moiety comprising an antigen-binding site and is linked to the C_n and (ii) a pharmaceutically-acceptable carrier.

10 The composition has been described above, and can comprise further compounds, such as therapeutic molecule associated with the C_n -Ab, among others as described above.

“Treat,” or variations thereof, when used in this context, refers to the reduction or elimination of the symptoms of a disease.

15 Any mammal can be a recipient of the administered composition according to this method. In one embodiment, the mammal is a human. In another embodiment, the mammal is a non-human mammal which provides economic, research, or esthetic utility to humans. Examples of such non-human mammals include, but are not limited to, cattle, horses, sheep, goats, monkeys, apes, rats, mice, dogs, and cats, among others.

20 In one embodiment, the disease is any disease which can be treated by a therapeutic molecule. The therapeutic molecule can upregulate or downregulate one or more metabolic processes in healthy cells, unhealthy cells, tumor cells, parasites, bacteria, or other infectious agents, in order to reduce or eliminate effects detrimental to the health of the mammal or increase effects beneficial to the health of the mammal
25 resulting from the metabolic process or processes. The disease can lead to such symptoms as one or more of damage to intracellular components; death or morbidity of cells; or failure of tissues, organs, or organ systems. The disease can be chronic or acute, and can arise from infection, exposure to agents in the environment, autoimmune response, or genetic defect, among others. In one embodiment, the disease is a cancer, by which is
30 meant a disease in which malignant cells arise within the mammal.

The composition can be administered either systemically or locally. Appropriate routes for systemic administration include, but are not limited to, intravenously, orally, nasally, or rectally, among others. Appropriate routes for local administration include, but are not limited to, subcutaneously or transdermally, among others. The inclusion of
5 an antigen-binding site in the C_n-Ab allows a systemic administration to provide localization to cells, tissues, organs, or organ systems wherein the antigen recognized by the antigen-binding site of the C_n-Ab is present.

The composition can be administered at any appropriate dosage which provides to the mammal an amount of the therapeutic molecule effective to treat the disease. In one
10 embodiment, the composition is administered at a dosage of from about 0.001 mg therapeutic molecule per kg body weight per day to about 1 g therapeutic molecule per kg body weight per day.

The composition can provide therapeutic benefit both by delivering a therapeutic molecule to a particular cell, tissue, organ, or organ system and by scavenging reactive
15 oxygen and other free radical species by antioxidant reactions at the C_n core.

The method can further comprise administering an adjuvant to the mammal, wherein the adjuvant dissociates the therapeutic molecule from the C_n-Ab. The adjuvant can be a chemical or an enzyme capable of specifically cleaving the linkage, if any, between the therapeutic molecule and the C_n-Ab. The adjuvant can be an energy source,
20 such as heat, light, ultrasound, or another energy source which emits energy sufficient to cleave a linkage between the therapeutic molecule and the C_n-Ab. The ordinary skilled artisan can determine other adjuvants as a matter of routine experimentation in light of the present disclosure.

25 The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the
30 present disclosure, appreciate that many changes can be made in the specific

embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1: Targeting Fullerenes using Recombinant Antibody Constructs

Background

Monoclonal antibody (MAb)-based procedures have traditionally been used in the diagnosis and therapy of human cancers. However, clinical use of these agents has met
5 with limited success due to drawbacks associated with this approach, e.g. heterogeneity of antigen expression, poor tumor penetration into solid tumors due in part to antibody size, and antigenicity of the antibodies (Roselli *et al.*, *In Vivo*, 7: 615-621 (1993); Berkower, *Current Opin. Biotechnol.*, 7: 622-628 (1996); Pullyblank *et al.*, *Brit. J. Surg.*, 84: 1511-1517 (1997); Panchagnula *et al.*, *J. Clin. Pharm. & Therapeutics*, 22: 7-19
10 (1997); Pancha, *Biochem. Pharmacol.*, 55: 247-252, (1998)). To circumvent these problems, a number of molecular approaches have been applied to reconfigure the conventional antibody structure into mouse:human chimeras, completely human antibodies or reshaped antibody fragments containing the antigen-binding portions of the original structure in a smaller and simpler (single-chain) format (Bird *et al.*, *Science*, 242: 423-426 (1988) [published erratum: *Science* 244: 409 (1989)]; Kipriyanov *et al.*, *Molec. Immunol.* 31: 1047-1058 (1994); Owens *et al.*, *J. Immunol. Methods*, 168: 149-165
15 (1994); McCartney *et al.*, *Protein Eng.*, 8: 301-314 (1995); Worn *et al.*, *Biochemistry*, 22: 13120-13127 (1998)). Single-chain antibodies (scfv, sfv), retaining the binding characteristics of the parent immunoglobulin (IgG), consist of the antibody V_L and V_H domains linked by a designed flexible peptide linker (Wels *et al.*, *J. Steroid Biochem. & Molec. Biol.*, 43: 1-7 (1992); Kurucz *et al.*, *Proc. Natl. Acad. Sci.*, 90: 3830-3834 (1993)). Furthermore, scfvs may be preferred in clinical and diagnostic applications currently involving conventional MAbs or Fab fragments thereof, since their smaller size may allow better penetration of tumor tissue, improved pharmacokinetics, and a
20 reduction in the immunogenicity observed with intravenously administered murine antibodies.

Among the few target antigens that are expressed at high levels in melanoma cells compared to normal tissue is the surface domain of a high molecular weight glycoprotein (gp240) found on a majority of melanoma cell lines and fresh tumor samples (Kantor *et al.*, *Hybridoma*, 1: 473-482 (1982)). Two murine antibodies (designated 9.2.27 and
30

ZME-018) recognizing different epitopes on this antigen have been previously isolated and described (Morgan *et al.*, *Hybridoma*, 1: 27-36 (1981); Wilson *et al.*, *Int. J. Cancer*, 28: 293-300 (1981)). The murine monoclonal antibody ZME-018 possesses high specificity for melanoma and is minimally reactive with a variety of normal tissues, making it a promising candidate for further study. Clinical trials examining the ability of this antibody to localize within melanoma lesions have demonstrated selective concentration in metastatic tumors (Macey *et al.*, *Am. J. of Physiologic Imaging*, 3: 1-6 (1988); Koizumi *et al.*, *Jap. J. Cancer Res.*, 79: 973-981 (1988)).

Successful development of tumor-targeted therapeutic agents is dependant, in part, on the site-specific delivery of therapeutic agents and also on the biological activity of the delivered agent. Monoclonal antibodies have been employed to impart selectivity to otherwise indiscriminately cytotoxic agents such as toxins, radionuclides, and growth factors (Williams *et al.*, *Cancer Res.*, 50: 974s-979s (1990); Rowlinson-Busza *et al.*, *Current Opin. Oncol.*, 4: 1142-1148 (1992); Wahl, *Cancer*, 73: 989-992 (1994)). The present study describes our investigation of a recombinant construct consisting of a single-chain analog of the antibody ZME-018 (cloned V_H and V_L domains) chemically fused to a chemically modified fullerenes. The recombinant antibody is engineered to contain a specific linker terminating in an amino acid molecule with properties specifically suitable for conjugation to the fullerene molecule. An example of the first such linkage will be a disulfide link between modified fullerene and a terminal cysteine amino acid.

Materials

The cDNA encoding antibody ZME-018 was amplified from hybridoma RNA obtained from hybridoma cells expressing the murine antibody using kits from Novagen (Madison, WI) and Invitrogen Corp. (Carlsbad, CA). The PCR reagents were obtained from Fisher Scientific (Pittsburgh, PA), the molecular biology enzymes were purchased from either Boehringer Mannheim (Indianapolis, IN) or New England Biolabs (Beverly, MA). Bacterial strains and pEt bacterial expression plasmids were obtained from Novagen (Madison, WI) and growth media was purchased from Difco Laboratories

(Detroit, MI). All other chemicals and reagents were either from Fisher Scientific or Sigma Chemical Co. (St. Louis, MO). Metal affinity resin (Talon) was obtained from Clontech Laboratories (Palo Alto, CA). Other chromatography resins and materials were from Pharmacia Biotech (Piscataway, NJ). Tissue culture reagents were from GIBCO BRL (Gaithersburg, MD). All DNA sequencing was performed at the M. D. Anderson Cancer Center Core Facility.

Methods

Cloning of the V_H and V_L domains of antibody ZME-018

10 Messenger RNA from murine hybridoma FMT 112 P2 expressing antibody ZME-018 (IgG2A) was isolated using the Invitrogen Fast Track kit and transcribed to cDNA with the Invitrogen Copy Kit using the specified conditions. Amplification of antibody light and heavy chain variable regions was carried out using the Novagen Ig-Prime kit with the mouse Ig-primer set. The PCR profile for light-chain amplification was as
15 follows: 30 cycles of 94°C x 1 min, 60°C x 1 min, and 72°C x 1 min terminated by a 5 min incubation at 72°C. For heavy-chain reactions, the identical conditions were used except that the annealing temperature was 50°C instead of 60°C. DNA amplified using this procedure was then cloned into the Invitrogen T/A cloning vector pCR II without further purification, transformed into *E. coli* XL1-Blue, and identified using blue-white
20 screening procedures. Positive clones (five each from the heavy-and light-chain libraries) were sequenced using the T-7 and SP6 promoter primers and antibody domains identified by homology to other immunoglobulin sequences.

Construction of genes encoding the single-chain antibody scfvMEL containing the terminal G4S linker with a terminal cysteine

25 A two-step splice-overlap extension PCR method (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)) was used to construct the single-chain antibody ZME-018 using light-and heavy-chain DNA clones as templates. Light-chain sequences were
30 amplified using the primers A (5'-GCTGCCCCAACCAGCC

ATGGCGGACATTGTGATG-3') and C (5'-
GCCGGAGCCTGGCTTGC(A/C)GCTGCCGCTGGTGGAGCCTTTGATC(A/T)CCAG
-3'), whereas heavy-chain DNA was amplified with the primers B (5'-
AAGCCAGGCTCCGGCGAAGGCAGCACCAAAGG CGAAGTGAAGGTT-3') and D
5 (5'- GCCACCGCCACCACTAGTTGAGGAGACTGT-3'). The PCR profiles for each set
of reactions were as follows: 30 cycles of 1-min denaturation at 94⁰C, 1 min annealing at
50⁰C, and a 1 min extension at 72⁰C, followed by a final 5-min incubation at 72⁰C. One-
tenth volume of each of these reactions were combined and used directly in a second
PCR with only primers A and D following the same reaction profile as before. The final
10 product was purified using GeneClean II (Bio 101, Vista, CA), digested with the
restriction enzymes Nco I and Spe I, and cloned into the T-7-based plasmid vector pET-
22b. The genes encoding scfvMEL and recombinant gelonin were fused together using
the splice-overlap extension PCR method with antibody and gelonin DNA as templates
and primers NbsphZME
15 (5'GGCGGTGGCTCCGTCATGACGGACATTGTGATGACCCAGTCT
CAAAAATTC-3'), primer NTXOM (5'-GGTGGCGGTGGCTCCGGTCTAGACAC
CGTGACG-3'), and primer XOMBAC (5'-
AAGGCTCGTGTGACCTCGAGTCATTAAGC TTAGGATCTTTATC-3') . Purified
PCR products were then purified and digested as before and cloned into the vector pET-
20 32a. Sequenced DNA clones were subsequently transformed into *E. coli* strain
AD494(DE3) pLys S obtained from Novogen for expression of the recombinant
construct.

Protein expression in E. coli

25 To express the recombinant antibody, bacterial cultures were incubated at 37°C in
2xYT growth medium with strong antibiotic selection (200 µg/ml ampicillin, 70 µg/ml
chloramphenicol, and 15 µg/ml of kanamycin) and grown until early log phase (A_{600} =
0.4-0.8). The cultures were then diluted 1:1 with fresh 2xYT medium containing the same
concentrations of antibiotics, and target protein expression was induced at 23°C by the

addition of 0.1 mM IPTG for 16-23 h. Induced bacterial cultures were then centrifuged and stored frozen at -80°C for later purification.

Frozen bacterial pellets from induced cultures expressing scFvZME/linker were thawed at room temperature and lysed by the addition of 1mg/ml lysozyme in 10mM
5 Tris-HCl, pH 8.0 for 30 min at 4°C. The bacterial lysates were then sonicated three times for 10 sec each with a cell disruptor and centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was transferred and saved on ice, and the sonication procedure was repeated with the cell pellet. Supernatants from the two lysates were then combined and ultracentrifuged at 40,000 rpm in a SS-34 rotor for 45 min at 4°C. The samples
10 containing only soluble protein were then filtered (0.22 µm pores), adjusted to 40 mM Tris-HCl with 1M Tris-HCl (pH 8.0), and then loaded at room temperature onto a Talon metal-affinity column pre-equilibrated with the same buffer. After loading, the column was washed with 3 column volumes of loading buffer, followed by a 5-column volume wash with 40 mM Tris-HCl pH 8.0, 500 mM NaCl, and 5 mM imidazole. Bound protein
15 was then eluted with 5 column volumes of buffer containing 40 mM Tris-HCl (pH 8.0), 500 mM NaCl and 100-200 mM imidazole. Fractions containing antibody/linker fusion were combined, quantitated, and dialyzed into 20 mM Tris-HCl (pH 7.2), 50 mM NaCl prior to digestion with enterokinase to remove the 6xHis tag using the procedure established by Novagen (Madison, WI).

20

Results

The genes encoding the antibody and G₄S-C linker fragments were linked together using a PCR-based method to construct a fusion in the antibody-linker orientation. The gene fusion gene was also C-terminal tagged with a hexahistidine
25 sequence and expressed in *E. coli* AD494(DE3) pLysS using the Novagen T-7-based expression vector pET-32b.

The antibody was constructed to encode the light chain variable region (V_L) at the N-terminus of the protein with an 18 amino acid flexible peptide linker (Stirpe *et al.*, *J. Biol. Chem.* 255: 6947-6953 (1992)) with the V_H C-terminus. The G₄S-C linker(Glycine-
30 Glycine-Glycine-Glycine-Serine-Cysteine) was positioned downstream of the V_H. We

chose this configuration for reasons involving the unhindered flexibility of the antibody-binding site. DNA-sequencing studies of the final fusion protein confirmed the sequence of the final product and that no errors had been introduced using this PCR method. In addition, DNA sequencing also confirmed that the target gene was inserted into the correct reading frame in the pET-32b vector.

The plasmid vector pET-32b containing the fusion gene was transformed into *E. coli* AD494(DE3) pLysS, and expression of the target protein was induced by the addition of IPTG. As seen in a coomassie-stained gel, a protein of the expected molecular mass (45 kDa) was induced. This protein was purified using IMAC resin, and the eluate was exposed to recombinant enterokinase (EK) to yield the final native fusion construct migrating as one band at 25 kDa. The fusion construct was also examined by Western blot using an anti-single-chain antibody.

Example 2: Preparation of a C_n-Ab associated with a radioisotope

A C_n can be associated with a therapeutic molecule comprising a radioisotope by any of a variety of techniques. In one technique, the C_n is fabricated in the presence of a radioisotopic metal, and the C_n forms with radioisotope atoms encaged therein. The fabrication process can comprise the high-temperature reaction of graphite impregnated with the radioisotope or the solution reaction of polycyclic aromatic molecules in the presence of the radioisotope in solution or suspension, among other techniques. C_n containing encaged radioisotope can then be purified from C_n not containing encaged radioisotope. C_n containing encaged radioisotope can then be attached to an Ab, such as by the techniques discussed in Example 1.

In another technique, the C_n is fabricated not in the presence of the radioisotope, by any appropriate technique, and thereafter the radioisotope can be loaded into the C_n by nuclear recoil.

Depending on the technique, an Ab can be attached to the C_n either before or after association of the radioisotope to the C_n.

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied
5 to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and
10 modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.